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PRINCIPAL INVESTIGATOR: John Teiber

CONTRACTING ORGANIZATION: The University of Texas Southwestern Medical Center
Dallas, TX 75390

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Teiber, John, F.

E-Mail john.teiber@utsouthwestern.edu

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14. ABSTRACT

The *P.aeruginosa* signaling and virulence molecule 3OC12 mediates inactivation of the lactonase paraoxonase 2 (PON2) and induces many immunomodulatory effects in host cells. Because PON2 rapidly inactivates 3OC12, we hypothesized that preventing PON2 inactivation by
3OC12 could be a viable therapeutic strategy to limit *P.aeruginosa* quorum signaling and thereby attenuate virulence. We demonstrated in both human and mouse primary cell types that PON2 is sensitive to 3OC12-mediated inactivation at concentrations of 3OC12 expected to be present near *P.aeruginosa* colonies during infection. We also discovered that 3OC12 is rapidly hydrolyzed intracellularly by PON2 to
3OC12-acid, which becomes trapped and accumulates within the cells. 3OC12 caused a rapid cytosolic pH decrease, calcium release and phosphorylation of stress signaling kinases. All of these effects were dependent upon PON2 activity. A potent PON2 inhibitor was also identified and treatment of cells with the inhibitor prevented 3OC12-mediated inactivation of PON2. The findings suggest the intracellular acidification is that proximal event that mediates both PON2 inactivation and many immunomodulatory effects of 3OC12. Thus, protecting cells from 3OC12-mediated acidification could be an important therapeutic strategy to attenuate *P.aeruginosa* quorum signaling and virulence in infected individuals.

15. SUBJECT TERMS
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II. Introduction

Mammalian paraoxonase 2 (PON2) is a ubiquitously expressed intracellular esterase that has been shown to efficiently hydrolyze, and thereby inactivate, the *P. aeruginosa* quorum sensing molecule 3OC12(1). This suggests that PON2 may be an important component of the innate defense which can disrupt bacterial quorum sensing, limiting the pathogenicity of the bacteria. We have previously shown that treatment of cells with 3OC12 results in a very rapid, and apparent Ca^{2+}-dependent, inactivation of PON2 hydrolytic activity and also a slower down-regulation of PON2 protein and mRNA(2). Thus, we hypothesized that PON2 is an important component of the innate defense against *P. aeruginosa* that acts by inactivating 3OC12, but that 3OC12 can inactivate/down-regulate PON2 through Ca^{2+} induction. If correct, prevention of PON2 inactivation/down-regulation could be a successful therapeutic strategy to attenuate *P. aeruginosa* virulence, rendering the bacteria more susceptible to antibiotics and clearance by the host immune response. Therefore the objectives of this study were to identify the mechanism and signaling pathways by which 3OC12 inactivates PON2 so they may be targeted by potential inhibitor drug candidates. To help established the physiological relevance of 3OC12-mediated down-regulation of PON2 we also sought to determine the sensitivity of PON2 to inactivation of its hydrolytic activity and down-regulation of its protein and mRNA by 3OC12 in primary cells relevant to *P. aeruginosa* infection and in vivo in mice. After demonstrating and characterizing PON2 inactivation and down-regulation by 3OC12 in mice, we proposed to demonstrate that PON2 activity can be protected from 3OC12-mediated inactivation in mice by compounds shown to prevent PON2 inactivation.

III. Results

Aim 1, Task 1

The objective of this task was to determine if 3OC12 treatment of cells caused changes in the post-translation modification of PON2. HEK PON2-GFP cells were treated with or without 100 μM 3OC12 (previously shown to inactivate > 90% of PON2 activity) and PON2 immunoprecipitated (IP). The IP PON2 was run on an SDS-PAGE gel and the PON2 band excised and analyzed for post-translational modifications by mass spectrometry at the Max Plank Institute of Biochemistry. Proteolytic digestion of the excised PON2 band with trypsin, chymotrypsin and GluC protease followed by mass spectrometry analysis, resulted in a 91% sequence coverage of PON2. The only modification identified was phosphorylation of serine 36 (S36). However, no difference in phosphorylation of S36 between PON2 from 3OC12 treated (inactivated) and untreated cells was observed. Because IP PON2 from 3OC12 treated cells is likely a mixture of active and inactivated enzyme, presence of phosphorylation on S36 from 3OC12 treated cells cannot be interpreted to indicate that this residue is refractory to dephosphorylation upon 3OC12 treatment. The effect of phosphorylation of serine 36 on PON2 activity was determined (see Aim 1, Task 3 below). It should be noted that the lack of identification of a post-translational modification on a protein by mass spectrometry analysis is not a definitive indication that such a modification does not exist.

Aim 1, Task 2

Phosphatase inactivation of PON2 - Our preliminary data included in the grant proposal suggested that dephosphorylation of PON2 may be the post-translational event that inactivates the enzyme. Therefore the ability of phosphatases to inactivate PON2 was tested. Lysates from EA.hy 926 cells over-expressing PON2 were treated with the purified calcium/calmodulin (CALM)-dependent phosphatase calcineurin (CN) and CALM (Sigma) according to the manufacturers protocol and then analyzed by HPLC for PON2 activity. No change in PON2 activity was observed (data not shown). The assay was repeated using increased concentrations of CN and/or CALM and incubation times, however no change in PON2 activity was observed. Isolated microsomes from the EA.hy 926-PON2 cells (which contain high levels of PON2) were also treated with CN + CALM. As with cell lysates, treatment with CN + CALM did not affect PON2 activity in the microsomal fraction.

Lysates from EA.hy 926 cells over-expressing PON2, and in a separate experiment lysates from A549 cells, were also treated with lambda phosphatase (New England Biolabs) according the manufacturers protocol and then analyzed for PON2 activity. No change in PON2 activity was observed. The findings suggest that

PON2 is either not a substrate for the phosphatases or that dephosphorylation does not modulate its activity. However, it is known that denatured proteins are generally more effectively dephosphorylated in vitro by the phosphatases than proteins that are treated under conditions designed to maintain the protein's natural state. Therefore, it is possible that under our assay conditions, i.e. conditions that do not denature PON2 so that its enzymatic activity can be assessed, the phosphatase may not be effectively dephosphorylating the native PON2.

Due to the lack of effect of phosphatases on PON2 activity alternative experimental approaches were tested in an effort to identify proteins that mediate PON2 inactivation. Calcium is suggested to be the proximal mediator of PON2 inactivation in intact cells. To determine if PON2 could be inactivated in vitro, cells were gently lysed in the presence of 1 mM calcium and incubated at 37^{o}C for increasing times. No inactivation of PON2 was observed. Furthermore, incubating cell lysates from untreated cells with lysates from cells treated with 3OC12 (presumably activating the proteins that mediate PON2 inactivation) resulted in no inactivation of PON2 in the untreated lysates. This suggested to us that, for PON2 inactivation to occur, PON2 and/or the signaling proteins must be properly localized in their natural environment in intact cells.

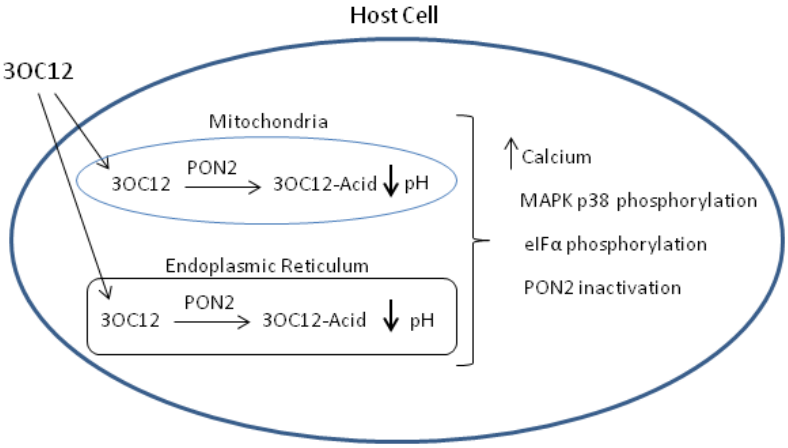
Identification of a PON2 interacting protein – As some initial experiments did not yield conclusive results, additional experimental approaches and experiments have been undertaken to identify the molecular mechanisms of 3OC12-mediated PON2 inactivation. The inability of phosphatases to inactivation PON2 in cell lysates and our extensive analysis of PON2 mutants (see Aim 1 Task 3 below) suggested that a post-translation modification of PON2 may not be the molecular event inactivating the enzyme. Thus, we hypothesized that 3OC12 treatment may initiate an interaction of PON2 with another protein(s) that renders PON2 inactivate. We initially tested this hypothesis by incubating cultured A549 cells treated with 3OC12, or left untreated, with the crosslinking agent formaldehyde. Formaldehyde is a small molecule and crosslinks proteins that are in very close association. Only in cells treated with both 3OC12 and formaldehyde was a band visible, of approximately 300 kD, on the PON2 immunoblot (Fig. 1A). Similar results were observed when cultured A549 cells were treated with the intracellular crosslinkers disuccinimidyl suberate (Thermo Scientific) which crosslinks proteins at primary amino groups or bis(maleimido)hexane (Thermo Scientific) which crosslinks proteins at sulfhydryl groups (data not shown). To further characterize the putative protein interaction of inactivated PON2, cell lysates from 3OC12 or acidic hanks balanced salt solution (HBSS) treated cells were incubated with the water soluble crosslinker bis[sulfosucinimidyl] suberate (BS^3; Thermo Scientific) per the manufactures instructions. BS^3 crosslinks proteins at primary amino groups. We had previously demonstrated that treatment of cells for 2 hrs with HBSS at pH 4.7 results in a 75% inhibition of PON2 activity, but does not alter PON2 protein levels (data not shown). HBSS (pH 7.2) has no effect on PON2. Only in cells treated with 3OC12 or pH 4.7 HBSS, followed by treatment with BS^3, was the ~300 kD band visible on the PON2 immunoblots (Fig. 1B). The same result was observed in EA.hy 926 PON2-GFP cells treated in the same manner (data not shown). Together the data demonstrate that 3OC12 mediates a close association of PON2 with another protein or protein complex. As discussed below, the ability of the low pH HBSS to inactivate PON2 and mediate crosslinking, similar to 3OC12, suggests that decreased intracellular pH may be a key proximal event that contributes to 3OC12-mediated PON2 inactivation.

We speculate that this PON2-protein interaction could be rendering PON2 hydrolytically inactivate. To identify the putative PON2 interacting protein, our approach is to IP the ~300kD BS^3 crosslinked complex with a GFP antibody, run the IP on an SDS-PAGE, cut out the band and send it to our mass spectrometry facility for analysis. Fig 1C demonstrates that we were able to successfully IP the ~300kD complex from EA.hy 926 PON2-GFP cells using the Dynabead magnetic bead technology (Invitrogen). We are in the process of scaling up the IP so that sufficient amounts of the PON2-protein complex are available for mass spectrometry analysis. Currently we have had problems getting consistent sufficient quantities of the IP PON2-complex and have been attempting to optimizing conditions and are also using HEK 293 PON2-GFP cells which express 3 fold higher levels of PON2 compared to the EA.hy 926 PON-GFP cells. An alternative IP method using the well established and commercially available Protein G or A Magnetic Beads may be employed if the Dynabead protocol cannot be optimized.

Discovery of a novel mechanism mediating biological effects, and possibly PON2 inactivation, in mammalian cells – The ability of low pH HBSS treatment of cells to inactivate PON2 and induce formation of the PON2-protein complex suggested to us the intracellular acidification may be a proximal event which contributes to PON2 inactivation. We tested this hypothesis and demonstrated that 3OC12 is rapidly

hydrolyzed intracellularly by PON2 to 3OC12-acid which accumulates within the cells resulting in a rapid intracellular pH decrease, calcium release and phosphorylation of stress signaling kinases (Scheme 1). The data from these studies and a description and interpretation of the findings are detailed in a manuscript that is currently under review in The Journal of Biological Chemistry. The complete manuscript including that data and figures and can be viewed in the appendix of this report and are not shown here. These findings extend beyond the mechanism of 3OC12-mediated PON2 inactivation and provide strong evidence of a common mechanism by which 3OC12 can elicit biological responses in host cell, many of which are thought to subvert host immune defenses. Thus, identification of this novel mechanism should greatly accelerate the understanding of how *Pseudomonas aeruginosa*, as well as other gram-negative bacteria, utilize acyl-homoserine lactones, such as 3OC12, to modulate host immune responses.

The PON2-mediated intracellular accumulation of 3OC12-acid and pH drop suggests that acidification within the endoplasmic reticulum and/or mitochondria (where PON2 is localized) may be the proximal event that leads to, or at least contributes to, PON2 inactivation. We are currently utilizing PON2 competitive inhibitors in cell culture studies to test the hypothesis that hydrolysis of 3OC12 to the 3OC12-acid is necessary for PON2 inactivation.



Scheme 1. Paraoxonase 2 mediates biological responses to 3OC12 in mammalian cells. 3OC12 rapidly enters the cells and distributes to the endoplasmic reticulum and the mitochondria where it is efficiently hydrolyzed by PON2 to the corresponding carboxy acid 3OC12-acid. The accumulation of 3OC12-acid results in a rapid drop in pH within the organelles, the proposed proximal event which leads to a rapid cytosolic calcium rise, phosphorylation of stress related kinases, PON2 inactivation and likely other biological responses.

Aim 1, Task 3:

Analysis of PON2 mutants – The objective of this task was to construct and analyze PON2 mutants in an effort to identify a mutant that resisted PON2-mediated inactivation. Recombinant PON2-GFP mutants were constructed and transiently transfected into HEK 293 cells. Amino acids capable of being phosphorylated were either mutated to alanine, which would resist modification (e.g. phosphorylation), or glutamic acid (E) or aspartic acid (D) which would mimic phosphorylation. Sites chosen for mutation were based primarily on findings from phosphorylation prediction programs that showed amino acid residues with a high likely hood of being phosphorylated. Additionally, based on published crystal structural data from PON1, residues predicted to be near the substrate binding or active sites were also considered for mutation. In addition to the hypothesis that a single post-translational event inactivates PON2, we also considered that multiple post-translational modifications may occur, both of which inactivate the enzyme. Therefore, we also made and tested double mutants. For the double mutants the serine 36 to glutamic acid modification was always included as this mutant is active (Table 1) and mass spectrometry analysis indicated this residue is phosphorylated. Transfected cells were either treated with 3OC12 (to initiate PON2 inactivation) or vehicle and cell lysates then tested for PON2 hydrolytic activity. For each mutant analyzed, a positive control that consisted of HEK 293 cells transiently transfected with wild type PON2-GFP and a negative control consisting of untreated cells were also analyzed. PON2 activity in lysates was determined by HPLC analysis of 3OC12 hydrolysis. Expression of the PON2 constructs was validated by Western analysis of the cell lysates. A summary of the results is given in the Table 1.

TABLE 1. Summary of analyses PON2 mutant constructs.

Mutation	Finding
S31A	Maintains activity. Still inactivated by 3OC12.
S31E	Maintains activity. Still inactivated by 3OC12.
S36A	Very Low activity. Possible small amount of inactivation.
S36E	High activity. Still inactivated by 3OC12.
S36D	High activity. Still inactivated by 3OC12.
S103A	Maintains activity. Still inactivated by 3OC12.
S165E	Maintains activity. Still inactivated by 3OC12.
Y192F	Maintains activity. Still inactivated by 3OC12.
T195A	Maintains activity. Still inactivated by 3OC12.
S208A	High activity. Still inactivated by 3OC12.
S221A	Maintains activity. Still inactivated by 3OC12.
S228A	Maintains activity. Still inactivated by 3OC12.
Y293A	Maintains activity. Still inactivated by 3OC12.
S331E	Little to no activity.
S331D	Little to no activity.
S331A	Little to no activity.
S334A	Maintains activity. Still inactivated by 3OC12.
S334E	Little to no activity.
S36E, S334A	High activity. Still inactivated by 3OC12.
S36E, S331D	No activity.
Y346F	Maintains activity. Still inactivated by 3OC12.

Mass spectrometric analysis indicates that PON2 is phosphorylated on S36. Correspondingly, the phospho-mimetic mutants S36E and S36D retained activity, whereas phospho-resistant mutant (S36A) was essentially devoid of activity (Table 1). Together the data provide strong evidence that phosphorylation of PON2 on S36 is required for hydrolytic activity. Interestingly the phospho-mimetic mutants were not inactivated by 3OC12 (Table 1), indicating that dephosphorylation of S36 is not the mechanism by which PON2 is inactivated by 3OC12 despite the apparent necessity for active PON2 to have this serine phosphorylated. The data suggest the possibility of a population of PON2 that is hydrolytically inactive (i.e. dephosphorylated S36) but has the potential to be rapidly activated by phosphorylation. If so, identification and activation of the kinase that phosphorylates PON2 on serine 36 may be a viable strategy to increase PON2 activity and the capacity of cells to inactivate 3OC12.

Subcellular localization of PON2 mutants – The effect of mutations of PON2 on their subcellular localization was determined. PON2-GFP mutants were transfected into HEK 293 cells and subcelluar localization was analyzed by confocal microscopy. Analysis of 5 mutants demonstrated that there were no alterations in their subcellular localization (Fig. 2). Given that none of the 5 mutants tested showed alteration in subcellular localization, it was decided that this analysis is only necessary when a mutant(s) is identified that resists 3OC12-mediated inactivation. Thus, to limit unnecessary expenditures mutants that are not resistant to 3OC12-mediated inactivation will not be analyzed for subcellular localization.

Aim 1, Task 4:

Treatment of cultured cells with signaling pathway inhibitors and activators - The primary objective of this task was to identify signaling proteins and pathways that mediate the inactivation of PON2 hydrolytic activity by use of protein and signaling pathways specific inhibitors and activators. An exhaustive screening of compounds has been performed. For these studies cultured cells are pretreated with commercially available

inhibitors or activators of signaling pathway proteins and then treated with 3OC12, or the ionophore A23187 which also inactivates PON2, to inactivate PON2. Concentrations and durations of 3OC12 and A23187 used were previously optimized and result in the inactivation of over 50 % of PON2. We also co-pretreated cells with a mixture of inhibitors to test the hypothesis that multiple events, mediated by multiple signaling proteins, inactivate PON2. The compounds tested were typically incubated with cells for 30-60 min before 3OC12 or A23187 treatment (unless indicated otherwise) at 2-10 fold the concentrations previously reported in the literature to inhibit or activate their target protein or signaling pathway. The inhibitors or activators were also always present in cell culture media during 3OC12 and A23187 treatments. A summary of the results is given in Table 2. W7, a CALM inhibitor, was the only compound identified that resulted in a consistent and nearly complete protection of PON2 from inactivation. CALM is calcium dependent and while this finding is consistent with our hypothesis of a calcium-dependent mechanism, this compound has recently been shown to have off target effects(3). Furthermore, we could not demonstrate a direct inactivation of PON2 with the CALM activator CALP1. W7 was also directly toxic to cells, resulting in rounding and detachment within approximately 45 min after treatment. Such toxicity and required high doses need to protect PON2, 50 μM may limit its usefulness in in vivo studies. Interestingly W7 is a weak base and this property has been suggested to contribute to off target (non-CALM) effects(3). Thus, it may be possible that W7 is protecting PON2 from inactivation by buffering the intracellular acidification resulting from the PON2 hydrolysis of 3OC12 to 3OC12-acid.

TABLE 2. Summary of effects of inhibitors and activators on PON2 activity alone or after 3OC12 or A23187 treatment.

Inhibitor or Activator	Primary Target	Finding
W7	Calmodulin Inhibitor	At ≥45 min pretreatment is toxic to cells. 20 min pretreatment at 50 μM protects PON2 from 3OC12 and A23187 in A549 and Hela cells.
CALP1	Calmodulin Activator	100 -150 μM had no affect on PON2 in A549 or A172 cells.
KN-93	CaM Kinase II Inhibitor	Small amount of PON2 protection, but not consistent.
STO-609	CaM Kinase Kinase Inhibitor	No PON2 protection or direct effects on PON2 in A549.
Tacrolimus	Calcineurin Inhibitor	Shows some PON2 protection in A549 and Hela cells, but protection is inconsistent. When DMSO stock solutions were used tacrolimus was not soluble in media.
Cyclosporin	Calcineurin Inhibitor	Similar results as with tacrolimus except lower levels of PON2 protection. Reports of poor cell permeability may limit usefulness.
Cypermethrin	Calcineurin Inhibitor	No PON2 protection in A549 or Hela cells.
CN inhibitory peptide	Calcineurin Inhibitor (membrane permeable)	No PON2 protection in A549 cells.
Okadaic Acid	Inhibitor of protein phosphatase isozymes PP1, PP2, PP4 and PP5.	2.5 μM No protection of PON2 in A549. OA sodium salt (10μM in A549), very minor (10%) protection.
HA10004	PKA, CaMKII, MLCK, PKC and PKG kinases inhibitor	100 μM no PON2 protection in A549 or Hela cells.
Staurosporine	PKA, PKG, CaMKII and MLCK kinases inhibitor	1 hr pretreatment. 1 μM no PON2 protection in A549s. 2.5 μM no protection A549.
Genistein	Tyrosine Kinases Inhibitor	No PON2 protection or direct effects on PON2 in A549 cells after 1-18 hr treatments.
Anisomycin	p38 kinase and c-Jun N-terminal kinase (JNK) activator	No PON2 protection or direct effects on PON2 in A549 cells after 1-18 hr treatments.
PD98059	ERK1/2 kinase	40 μM, 1 hr – no PON2 protection in A549 cells
SB216763	Glycogen synthase 3b kinase inhibitor	A549 cells 25 μM (a lot of precipitate), 1 hr- About 25% protection of PON2 5 μM (no precipitate), 1 hr – about 40% protection of PON2 1-5 μM no protection against A23187 5 μM no protection against HBSS pH 4.7 mediated PON2 inactivation
SB203580	p38 kinase inhibitor	20 μM, 1 hr- No protection of PON2.
MIX	Mixture of indicated	A549 cells ERK1/2 (20 μM), GSK3b (1 μM), p38 (10 μM), Staurosporine (2.5 μM), Genistein (125 μM), Cypermethrin (20 μM), CAIP 10 μl in 500 μl. FINDING: About 20% protection of PON2
DAPK inhibitor	Death associated protein	20 uM, 1hr- No protection of PON2 in A549 cells

	kinase inhibitor	
SP600125	JNK kinase inhibitor	4 μM, 1 hr- No protection of PON2 in A549 cells
Serum Starvation		Overnight. No effect on PON2 activity in A549 cells.
AG-490	JAK2, HIF1 inhibitor	25 μM, 3 Day treatment in A549 cells, 25% increase in PON2 activity.
CAY10585	HIF1 inhibitor	Toxic to cells. 2-3 day treatment no effect on PON2 activity in A549 cells.
Dithiothreitol	Reducing agent that induces PON2 expression	2 mM 30 - 60 min, no protection of PON2 from 3OC12 inactivation.
TQ416	Unknown	15 min pretreatment at 10 μM - About 90% protection of PON2 inactivation

After treatment and inactivation of PON2 in cultured cells with 3OC12, PON2 activity will recover to roughly 50% of initial activity levels within 2 hours (while PON2 protein levels are still declining) if cells are left in culture(2). To identify signaling proteins or pathways that mediate re-activation of PON2, the ability of specific inhibitors to block PON2 reactivation of in EA.hy 926 PON2-GFP cells was determined. Staurosporine (broad spectrum kinase inhibitor) tacrolimus (calcineurin inhibitor), HA10004 (broad spectrum kinase inhibitor) or staurosporine and calciuneurin together were unable to prevent the reactivation of PON2 after its initial inactivation with 3OC12 (data not shown).

It was recently reported that a class of triazolo[4,3-a]quinoline compounds were able to potently inhibit a range of immediate 3OC12 initiated biological responses, including those tested in our manuscript (see appendix and Scheme 1 above), in cultured cells(*4*). Given our findings that many of these effects were PON2-dependent (Scheme 1), we hypothesized that these triazolo[4,3-a]quinoline compounds were inhibiting PON2 and thereby preventing the intracellular 3OC12-acid accumulation and acidification-mediated effects. Therefore, the most potent triazolo[4,3-a]quinoline compound (TQ416) was recently purchased (ChemDiv) and tested for its ability to inhibit PON2. In HEK PON2 cell lysates treated with 10 μM TQ416, 3OC12 (75 μM) hydrolysis (PON2 activity) was inhibited by 100 % (data not shown). We then treated human bronchial epithelial cells (HBEC) with TQ416 or TQ416 followed by 100 μM 3OC12 for 10 minutes and analyzed cell lysates for PON2 activity. TQ416 alone had no effect on PON2 activity, whereas TQ416 almost completely prevented 3OC12-mediated PON2 inactivation. While these findings are somewhat preliminary, experiments are currently being repeated and extended, they suggest that TQ416 is a potent PON2 inhibitor and the PON2-mediated hydrolysis of 3OC12 and intracellular acidification is the proximal event that mediates PON2 inactivation by 3OC12. Thus, preventing intracellular acidification could be a therapeutic strategy to protect PON2 from inactivation and also prevent many of the potential immunomodulatory effects of 3OC12 in the host.

A potent inhibitor of PON2 hydrolytic activity has never been described. Potent and specific enzyme inhibitors are invaluable tools which can be used to study enzyme functions and properties. Our finding suggests that TQ416 is a potent PON2 inhibitor which can be potentially be used in vitro, in cell culture and in vivo to identify the role PON2 plays in 3OC12 quorum signaling and host cell responses. Additionally, this inhibitor could be used to delineate the role between PON2's different protective functions, i.e. ester/lactone hydrolysis and antioxidant/anti-apoptotic functions. Thus, we will continue experiments to fully characterize TQ416 and develop it as a probe to delineate the role PON2 plays in 3OC12-mediated quorum signaling and host cell immunomodulation in cell cultures and potentially in vivo.

Inhibitory RNA studies – Our previous studies had suggested that cytosolic calcium rise is the proximal event that mediates PON2 inactivation. These findings and the finding that the CALM inhibitor W7 could protect PON2 from inactivation suggested that CALM could be mediating the inhibition of PON2. CALM is a calcium dependent protein that mediates a broad range of signaling events initiated by calcium, including activation of kinases and the phosphatase CN. Therefore, in an attempt to verify findings with W7 we initiated studies to down-regulate CALM and CN to assess their role in PON2 inactivation. To block CALM cells were transfected with three different CALM targeting siRNA's or a dominant negative calmodulin (dnCALM) plasmid. The dnCALM is biologically inactive and will bind to the endogenous CALM, blocking its activity. To ensure the most complete inactivation of CALM possible, in some studies cells were transfected with both CALM siRNAs and dnCALM. A representative Western analysis of CALM expression in cells transfection and 3OC12 treatment is shown in Figure 3A. Transfection with the CALM siRNA resulted in a slight knockdown of dnCALM (Fig 3A). Transfection with the dnCALM resulted in a high level of its expression and also appeared to decrease the expression of endogenous CALM, Figure 2A, lanes 6-9. Blocking of CALM activity after transfecting cells with CALM siRNAs or co-transfecting with CALM siRNAs and dnCALM did not protect PON2

from 3OC12-mediated activation (Fig 3C). This finding concurs with our finding the CALM activator CALP1 did not affect PON2 activity (TABLE 2) and suggests that CALM is not involved in the 3OC12-mediated PON2 inactivation. Interestingly, blocking of CALM alone appeared to increase PON2 activity in the cells (Fig 3B and C), suggesting basal CALM activity or functions may partly inactivate PON2 or decrease its expression. These studies are currently being repeated to verify the findings. CN knock down studies are also in progress to verify the role, if any, that this phosphatase has in regulation PON2 activity.

Aim 2, Task 1

The objective of Aim 2 task 1 was determine the sensitivity of PON2 to 3OC12-mediated inactivation and down-regulation in primary cell types to help establish the relevance of PON2 inactivation in patients infected with *P. aeruginosa*. 3OC12 concentrations ranging from 0.02 μM to > 6 μM have been detected in sputum samples from individuals with pulmonary *P. aeruginosa* infections(*5*). However, concentrations near *P. aeruginosa A* colonies and biofilms are likely to be significantly higher due to dilution into a large volume of sputum and enzymatic and spontaneous 3OC12 hydrolysis. Concentrations up to 600 μM 3OC12 have been measured in *P. aeruginosa* biofilms in vitro(*6*). Thus, concentrations of 3OC12 used in our studies would be expected in the region of *P. aeruginosa* colonies. Primary human cells or primary cells obtained from the mice were cultured and treated with 3OC12. We have previously extensively characterized the inactivation and down-regulation of PON2 in A549 cells so these cells were used as a positive control. Primary human aortic endothelial cells (HAEC) were as sensitive to rapid PON2 inactivate as the A549 cells, exhibiting over 50 % inactivation after a 10 min treatment with 25 μM 3OC12 (Fig 4A). PON2 in primary mouse peritoneal macrophages and lung cells was also inactivated by 3OC12, however these cells were not as sensitive as the HAEC cells (Fig 4A). It should be noted that the primary cells obtained from the mouse lungs are grown for two weeks before use and cultures contain mostly fibroblasts, although a small number of epithelial cells were observed in the cultures. Primary cells were also treated with the ionophore A23187, which presumably inactivates PON2 by increasing cytosolic calcium. PON2 in both HAEC and mouse lung cells were very sensitive to inactivation by A23187. Because 3OC12 can induce apoptosis in many cell types and this could help favor *P. aeruginosa* persistence, we tested the sensitivity of HEAC cells to 3OC12-mediated toxicity. Even the lowest concentration of 3OC12 tested, 6.25 μM, was able to significantly decrease the viability of the HAECs (Fig 4C).

Our initial studies in HBECs indicated that PON2 was less sensitive to 3OC12-mediated inactivation after a 10 minute treatment, the time point used above, compared to the A459 and HAEC cells (data not shown). However the data was variable and is not shown here and is currently being retested. Therefore we analyzed PON2 inactivation in the HBECs after a 20-120 min treatment with 3OC12. The 20 min treatment would precede and decrease in PON2 expression and predominantly reflect the rapid, putative post-translational modification of PON2, whereas at longer time points both decrease of PON2 activity in the cell is likely due to the inactivation of PON2, decrease expression and potentially protein degradation. After a 20 min treatment with 3OC12, PON2 was sensitivity to inactivation in the HBECs (Fig 5A). Increase the time of 3OC12 treatment significantly increased the sensitivity of PON2 to inactivation/down-regulation (Fig 5B). Even a 3OC12 concentration as low as 12.5 μM could significantly inactivate/down-regulate PON2 when cells were treated for 2 hrs (Fig 5B). The findings demonstrate that PON2 in primary cell types expected to come in contact with *P. aeruginosa* during infection are sensitive to 3OC12-mediated inactivation. However, there is a significant different in sensitivity to 3OC12 depending upon the cell type. We are currently continuing extending these studies and also determining the sensitivity of PON2 mRNA and protein to down-regulation by 3OC12 in primary cell types. We have recently purchased a commercially available PON2 ELISA kit and are in the process of verifying its sensitivity for quantifying PON2 in the cell lysates. This ELISA should make quantification of PON2 protein more rapid and accurate that our previously used semi-quantitative Western analysis method.

As described in Aim 1 above, and in the manuscript attached in the appendix, we demonstrated that 3OC12 mediates a PON2-dependent intracellular accumulation of 3OC12-acid, pH drop and calcium release. We currently hypothesize that the intracellular acidification is the proximal event which at lease contributes to PON2 inactivation. To help demonstrate the physiological relevance of this novel mechanism, these biological measures were determined in the primary HBECs. Treatment resulted in a rapid and extensive intracellular accumulation of 3OC12 (Fig 6A) and also a minor drop in cytosolic pH (Fig 6B). A concentration of 50 μM 3OC12 caused a substantial rise in cytosolic calcium, similar to the phospholipase C activator m3M3FBS

(Calbiochem) which mediates release of calcium from the ER (6C). The data provide evidence that the PON2-dependent acidification mediates biological responses to 3OC12 in primary cells, similar to those demonstrated in cell lines (see appendix).

Aim 2 Task 2

The objective of Aim 2 task 2 was to characterize the ability of 3OC12 to inactivate and down regulate PON2 in mice. Our previously developed technique to deliver 3OC12 as an aerosol with the Microsprayer to mice lungs was used. Before initiation of experiments we demonstrated that evan's blue dye (0.25%) could be added to the solution to be administered so that the sections of lung that were exposed to the 3OC12 could be easily visualized. Thus, only sections of lung exposed to 3OC12 were analyzed for PON2 activity. Treatment of wild type (WT) mice lungs with 200 μM 3OC12 for 30 minutes did not result in a significant decrease in PON2 activity in the mouse lung tissue (Fig. 7).

The lack of PON2 inactivation in the treated mice suggested that PON1, which also hydrolyzes 3OC12 and is expressed at high levels in the blood, may be present in the lung and possible lung cells in sufficient amounts to inactivation 3OC12. Thus, experiments were repeated in PON1 KO (KO) mice.PON2 activity levels were determined in tissue samples from WT and KO mice. There were no differences in PON2 activity levels in the 4 tissues tested (lung, liver, kidney and brain) comparing the WT and KO mice (n=3 per group). PON1 is most highly expressed in the liver and the KO mice livers had no detectible paraoxonase activity. Thus, hydrolysis/inactivation of 3OC12 by PON1 will not occur in the KO mice. As previously discussed, 3OC12 has low solubility in aqueous solutions. To overcome this, so that mice can be administered a larger dose of 3OC12, 3OC12 was dissolved in a 30% solution of propylene glycol (PG). We found that 3OC12 was soluble in the 30% PG solution up to 600 μM, much higher than the 200 μM solution administered to mice in the previous experiment (Fig 7). Therefore, 50 μl of a 600 μM solution of 3OC12 in 30% PG was administered to the KO mice lungs by microsprayer (n=5 mice). Control mice (n=4) received only 30% PG. Thirty minutes later the lungs were removed and analyzed for PON2 activity. There was no difference in PON2 activity in the control lungs compared to the 3OC12 treated lungs (data not shown). We repeated this same experiment (control mice n=5; 3OC12 treated mice n=5) except the lungs were harvested after 20 hours instead of 30 minutes. Again there was no difference in PON2 activity in the control lungs compared to the treated lungs (data not shown).

The reason for the lack of inactivation of PON2 in our in vivo studies in not clear. Studies in primary cells suggest sensitivity of PON2 to inactivation in vivo. However, there are differences in sensitivity to inactivation depending upon cell type. PON2 in some cell types in the mouse lung may be relatively insensitive to inactivation by 3OC12. Despite the relatively high concentration of 3OC12 administered to mice lung, it is possible the total amount of 3OC12 is not enough to result in exposure to the large number of cells within the tissue. In typical cell culture experiment in which 3OC12 administered at 100 μM, the ratio of the total amount of 3OC12 to mg of culture cells is approximately 800 nmol/mg of cells. In the mouse lung exposure studies the ratio of 3OC12 to total mouse lung tissue (dry weight) is approximately 15 nmol/mg of lung. This much lower ratio of 3OC12 to cells in vivo, combined with the likely rapid hydrolysis of 3OC12 in lung by PON2, will likely result in much lower exposure of cells in vivo to 3OC12. To overcome this low likely low exposure to 3OC12 in our experimental approach, we will inject high concentrations of 3OC12 intravenously (through tail vein). This should result in distribution of higher levels of 3OC12 throughout the lung, as well as other highly perfused tissues. Tail vein administration of high concentration of 3OC12 has previously been successfully performed to demonstrate 3OC12 biological effects on NFkB activity in vivo(*7*). We have amended our animal protocol to include tail vein administration of 3OC12 to mice and it is currently under review by IACUC. Experiments will begin as soon as the amended protocol is approved by IACUC and ACURO.

IV. Key Research Accomplishments

- Discovered a novel PON2-dependent intracellular acidification mechanism by which 3OC12 elicits biological responses, putatively including PON2 inactivation, in mammalian cells. This mechanism likely mediates many of the previously identified, yet mechanistically unexplained, biological effects in host cells and is a significant advance in the understanding of the *P. aeruginosa*-host interactions.

- Demonstrated that inactivation of PON2 by 3OC12, and also an acidic environment, induces an interaction between PON2 and a protein (or protein complex) of approximately 300 kD. Such an interaction of PON2 has never been described and suggests a potential novel mechanism of PON2 regulation, including 3OC12-mediated inactivation.

- Identified serine 36 as a phosphorylated residue on PON2 that is required for hydrolytic activity. Suggests PON2 hydrolytic activity can be rapidly modulated by phosphorylation. Identification of the kinase that mediates this phosphorylation may be a therapeutic target.

- Demonstrated that primary human aortic endothelial cells and bronchial epithelial cells are sensitive to 3OC12-mediated calcium release and PON2 inactivation. Provides strong evidence that PON2 can undergo 3OC12-mediated inactivation in *P. aeruginosa* infected tissues.

- Identified an inhibitor of cellular 3OC12-mediated PON2 inactivation and PON2 hydrolytic activity. This is the first inhibitor of PON2 described and could be a very valuable tool used to characterize the role PON2 plays in 3OC12 bacterial quorum signaling and 3OC12-mediated host cell immunomodulation.

V. Reportable Outcomes

Submission of a manuscript entitled, “A novel paraoxonase 2-dependent mechanism mediating the biological effects of a *Pseudomonas aeruginosa* virulence molecule” to The Journal of Biological Chemistry for publication.

VI. Conclusion

During the course of the first year much progress has been made with respect to successfully carrying out many of specific aims of the proposed research in addition to making novel unanticipated discoveries. Our studies in human primary cells types indicate that PON2 is quite sensitive to 3OC12-mediated inactivation. *This suggests that PON2 inactivation is likely to occur in the proximity of P. aeruginosa colonies in infected individuals and supports the proposition that prevention of PON2 inactivation could be a therapeutic strategy to limit P. aeruginosa quorum signaling and virulence.*

We initially hypothesized that 3OC12-mediated calcium release is the proximal event that activates a signaling pathway that results in a PON2 post-translational modification (putatively phosphorylation) that inactivates the enzyme. After an exhaustive characterization of PON2 mutants and potential inhibitors and activators of cell signaling pathways, the data suggests that PON2-dependent 3OC12 hydrolysis and intracellular acidification is the proximal event that mediates PON2 inactivation. In cell lines, but not yet primary cell types, we have demonstrated the 3OC12-mediated calcium release is dependent on hydrolysis of 3OC12 by PON2. Thus, calcium release, resulting from acidification may, still be a critical event in the pathway that leads to PON2 inactivation. However, we have previously shown that PONs are susceptible to inactivation in low pH conditions(*8*) , therefore the drop in pH within the ER and mitochondria may be directly inactivating PON2. We are currently characterizing the direct effect of lower pH on PON2 inactivation. Using crosslinking techniques, we have also discovered the 3OC12 mediates a close PON2 interaction with another protein or protein complex. This is likely downstream of the proximal signaling events. We are currently in the process of identifying this interacting protein. Once identified, we will initiate experiments to determine if its 3OC12-mediated association with PON2 causes PON2 inactivation. Regardless the mechanism that inactivates PON2, *the finding that acidification is likely the proximal event in PON2 inactivation indicates that prevention of acidification could be a viable therapeutic strategy to block PON2 inactivation and down-regulation.* Furthermore, as we have shown, PON2-dependent intracellular acidification can mediate calcium release and activation of stress kinases in cell lines. *Therefore, in addition to blocking PON2 inactivation, preventing cellular acidification may also be an important therapeutic strategy to prevent host cell effects of 3OC12 that undermine the host’s immune defenses.* Given the great of potential of this novel intracellular acidification

mechanism to impact the understanding of host-pathogen interactions, as well as to be a viable therapeutic target, we will continue to explore and delineate the details of this mechanism and it relevance in primary cell types.

We also appear to have discovered, for the first time, a potent inhibitor of PON2 hydrolytic activity. It is anticipated that this inhibitor will useful to study fundamental endogenous biochemical functions and properties of the enzyme. In addition the inhibitor is also anticipated to be a very valuable tool which can be used to delineate the roles that both intracellular acidification and PON2 play in 3OC12-mediated quorum signaling and host cell immunomodulation in cell cultures and potentially in vivo.

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VII. Appendix

A novel paraoxonase 2-dependent mechanism mediating the biological effects of a *Pseudomonas aeruginosa* virulence molecule[*]

Sven Horke[§†], Junhui Xiao[‡†], Eva-Maria Schütz[§], Gerald L. Kramer[‡], Petra Wilgenbus[§], Ines Witte[§], Ulrich Förstermann[§] and John F. Teiber[‡1,]

[†]These authors contributed equally to the work

[§]Department of Pharmacology, University Medical Center of the Johannes Gutenberg University Mainz, Obere Zahlbacher Str. 67, 55131 Mainz, Germany

[‡]Department of Internal Medicine, Division of Epidemiology, The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

[*]Running Title: *A novel mechanism of P. aeruginosa biological effects*

[1]To whom correspondence should be addressed: John F. Teiber, Department of Internal Medicine, Division of Epidemiology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-8874, USA, Tel.: 214 648 2454; Fax: 214 648 7893; E-mail: john.teiber@utsouthwestern.edu

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Background: The bacterial signaling molecule 3OC12 elicits immunomodultory responses in host cells through uncertain mechanisms.
Results: Human paraoxonase 2 hydrolyzed 3OC12 intracellularly to its hydroxyl acid, resulting in intracellular acidification, calcium release, and kinase activation.
Conclusion: Paraoxonase 2 mediates cellular responses to 3OC12 via a non-receptor mechanism in host cells.
Significance: Elucidation of host-pathogen interactions will help identify novel therapeutic strategies.

ABSTRACT

***Pseudomonas aeruginosa* produces *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3OC12), a crucial signaling molecule that elicits diverse biological responses in host cells thought to subvert immune defenses. The mechanism mediating many of these responses remains unknown. Here we demonstrate that 3OC12 is rapidly hydrolyzed intracellularly by the lactonase paraoxonase 2 (PON2) to 3OC12-acid, which becomes trapped and accumulates within the cells. 3OC12 caused a rapid cytosolic pH decrease, calcium release and phosphorylation of stress signaling kinases. All of these effects were dependent upon PON2 activity. Our results indicate a novel, PON2-dependent intracellular acidification mechanism by which 3OC12 can mediate its biological effects.**

Pseudomonas aeruginosa (*PA*) is a common pathogen causing serious infections in immunocompromised and ill individuals due to the bacteria's ability to evade host immune responses and acquire antibiotic resistance(1). Many gram-negative bacteria, including *PA*, produce acyl-homoserine lactone (AHL) signaling molecules which regulate the cell-density-dependent expression of virulence factors in a process termed quorum sensing (QS)(1). The AHL *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12) is a key *PA* QS signal that has been shown to be necessary for biofilm maturation and full expression of virulence in *PA* animal infection models(2;3). Concentrations of over 6 μM 3OC12

have been detected in the sputum of individuals with pulmonary *PA* infections(4), suggesting active 3OC12 signaling in the human disease as well.

3OC12 also elicits a multitude of biological responses in mammalian cells(5). 3OC12 induces chemotaxis in neutrophils(6). However, neutrophils, as well as most other cell types, are sensitive to 3OC12-mediated apoptosis(6;7). Induction of a range of pro-inflammatory cytokines and mediators by 3OC12 has been reported in fibroblasts, keratinocytes, epithelial, and endothelial cells, as well as in vivo(5). Conversely, 3OC12 inhibited lipopolysaccharide (LPS) induction of pro-inflammatory mediators in macrophages, fibroblasts, epithelial cells and in vivo by repressing nuclear factor κ-light chain enhancer of activated B-cells (NF-κB) signaling(8). In antigen-stimulated T-lymphocytes, 3OC12 inhibits cell proliferation and production of interferon-γ and IL-4, critical regulators of immunity(5;9). Interestingly, 3OC12 induced a Ca^{2+}-dependent NO production via the G-protein coupled taste receptor T2R38 in sinonasal epithelial cells, mediating direct antibacterial effects(10). These diverse responses suggest that 3OC12 acts through multiple, and cell type dependent, mechanisms.

The biological effects of 3OC12 on mammalian cells typically occur at concentrations of 5-100 μM(5). 3OC12 concentrations ranging from 0.02 μM to > 6 μM were detected in sputum samples from individuals with pulmonary *PA* infections(4). However, concentrations near *PA* colonies and biofilms are likely to be significantly higher due to dilution into a large volume of sputum and enzymatic and spontaneous 3OC12 hydrolysis. Concentrations up to 600 μM 3OC12 have been measured in *PA* biofilms in vitro(5). Thus, 3OC12 concentrations of 100 μM or more in the vicinity of *PA* colonies is likely.

Delineating the role of 3OC12 in *PA* pathogenicity has been difficult due to the lack of an understanding of the mechanisms by which 3OC12 modulates host cell immune functions. 3OC12 does not act through immune pattern recognition receptors such as Toll-like receptors and nucleotide binding, oligomerization domain-like receptors(11). In sinonasal epithelial cells the T2R38 receptor mediated a rapid Ca^{2+} and NO release by 3OC12, however T2R38 likely only mediates responses in upper respiratory cell types(10). Due to its lipophilicity, 3OC12 rapidly enters mammalian cells(9). 3OC12 can interact with nuclear hormone peroxisome proliferator activated receptor (PPAR) transcription factors, resulting in increased cytokine expression(12). However, such effects are relatively slow, occurring at ≥ 6 hours after 3OC12 treatment. Many effects of 3OC12, such kinase activation, occur within 5 minutes of treatment(11), a timeline preceding any gene expression. Other mechanisms of 3OC12-mediated effects have been proposed such as disruption of protein interactions and functions through intercalation into plasma membranes or covalent protein modification(13;14). However, evidence for such mechanisms is lacking and the mechanism(s) mediating many cellular responses to 3OC12 remains unknown.

The paraoxonase (PON) family of mammalian esterases, PON1, PON2 and PON3 hydrolyze AHLs to their ring-opened biologically inactive carboxylic acid counterparts(15). PON2 is expressed intracellularly, is widely found in mammalian tissues and cell types and very efficiently hydrolyzes 3OC12 to 3OC12-acid (15-17). Independent of its hydrolytic activity, PON2 also has antioxidant and antiapoptotic activity and can protect cells from endoplasmic reticulum (ER) stress, including ER stress induced by 3OC12(17-19). Such findings have led investigators to suggest that PON2 may be an important component of the innate defense by interfering with bacterial QS and attenuating 3OC12-mediated effects on host cells.

Here we identify a unique mechanism by which PON2 also mediates biological effects of 3OC12. We demonstrate that PON2 catalyzes the rapid hydrolysis of 3OC12 to 3OC12-acid, resulting in intracellular accumulation of 3OC12-acid, cytosolic acidification, calcium release and phosphorylation of p38 and eIF2-α. Thus, PON2 may be a double edged sword, disrupting bacterial QS and protecting from ER stress while simultaneously promoting many of the immunomodulatory effects of 3OC12 in host cells.

EXPERIMENTAL PROCEDURES

Cells - Generation and culturing of stable PON2 / PON2-H114Q overexpressing EA.hy 926

(EA.hy) cells and PON2 overexpressing HEK cells has been described(17;20). HUVECs, phenol red-free endothelial cell basal medium and endothelial cell growth medium supplement mix were purchased from PromoCell and HUVECs cultured as recommended by the supplier.

PON2 activity - For PON2 activity cell lysates were processed and analyzed for rates of 3OC12 hydrolysis by HPLC as previously described(15). PON2 activity is expressed as units per mg of cell lysate. One unit = 1 nmol of 3OC12 hydrolyzed per minute.

Intracellular 3OC12-acid determination - Cells were seeded in 24 well plates. Eighteen hours later, when cells were approximately 75% confluent, they were treated with 0.5 ml of whole growth media containing 25 µM 3OC12 and placed back in the cell culture incubator. At given times the cells were rinsed once with PBS and extracted with 100 µM cold acetonitrile containing 25 µM *N*-dodecanoyl-L-homoserine lactone as the internal standard. Extracts were centrifuged for 1 min at 14,000 x *g* and analyzed by HPLC as previously described(15). The concentration of 3OC12-acid in each sample was calculated from peak areas using a standard curve generated from 3OC12-acid standards. The 3OC12-acid was prepared by incubating 3OC12 in 5 mM NaOH for 2 hrs. Complete hydrolysis of 3OC12 to the 3OC12-acid was confirmed by HPLC analysis. Protein concentrations were determined by lysing in RIPA buffer, briefly sonicating and analyzing lysates using the BCA method (Thermo Scientific).

Cytosolic pH and Ca^{2+} measurements - For determination of pHi and $[Ca^{2+}]_c$ in the plate reader format, cells were seeded at 25,000 per well in black 96-well Costar plates and treated the following day. All treatments were performed at 37°C. For pHi determination cells were loaded with 2 µM BCECF-AM (Life Technologies) in 100 µl of whole growth media for 40 min, washed once with hank's balanced salt solution containing Ca^{2+} and Mg^{2+} (HBSS), and incubated for an additional 15 min in HBSS. Cells were washed again, treated with 25 µM of 3OC12, or an equivalent volume of DMSO as the control, in 100 µl of HBSS and fluorescence was measured using a Synergy HT fluorometric plate reader (Bio-Tek) with excitation wavelengths set at 485 nm and 360 nm and emission detected at 528 nm. The calibration of pHi was performed as previously described(21). Briefly, cells were treated with high KCl buffers (with pH values ranging from 6.2-7.5) containing nigericin. Once pHi equilibrated, fluorescence was measured at 528 nm after excitation at 485 nm and 360 nm. The pHi was determined from a calibration curve by plotting the 485/360 ratio observed against the buffer pH.

Intracellular Ca^{2+} was determined as previously described with minor modifications(17). Cells plated as described above were loaded with 4 μM Fluo-4 AM (Life Technologies) in medium containing 20 mM Hepes and 2.5 mM probenicid at 37°C for 45 min and then washed 3 times in HBSS. Fluorescence was measured for 5 minutes on the plate reader with excitation and emission wavelengths of 480 nm and 530 nm, respectively, and then treated with $HBSS^{++}$ containing DMSO or 25 µM 3OC12. Fluorescence was measured for 25 minutes at 37°C. Fluorescence values measured for both pHi and Ca^{2+} from DMSO treated cells were subtracted from values measured after 3OC12 treatment.

Confocal microscopy - EA.hy cells were seeded at $1x10^4$ in 4-well slide cover glass-I chambers (Greiner Bio-One). The next day, cells were loaded with SNARF-4F and Fluo4-AM (5 and 1 µM, respectively; Life Technologies) in Krebs buffer (Noxygen) at 37°C for 1 hour. After brief washes, chambers were loaded on a 37°C / 5% CO_2 incubator device mounted on a confocal Zeiss LSM710 laser-scanning microscope. Upon exposure to 3OC12, cells were immediately imaged with a EC Plan-Neofluar 20x/0.50M27 objective, 4.07 µsec pixel dwell, 54 µm pinhole and emission wavelengths of 493-516 nm (Fluo-4AM); SNARF-4F was calculated as ratio of acidic (581-601nm) / basic (650-738nm) fluorescence units. Thus, increased ratio indicates acidification. Using ZEN2009 software (Zeiss), the fluorescence intensities at every time point were recorded for approximately 50-100 cells per visible field and transferred to GraphPad prism software (GraphPad Software Inc.) for evaluation and data processing.

Western blotting - All lysates were produced in the presence of PhosphoStop phosphatase inhibitor (Roche). The PON2 antibody and immunoblotting has been previously described(17). Antibodies against eIF2α / phospho-eIF2a (Ser51) and p38 (Thr180/Tyr182) were from Cell Signaling and used as recommended by the supplier. Anti-GAPDH (clone 6C5; Santa Cruz) and mouse-anti-Tubulin Ab2 (Dianova) were used at 1:5000. HRP-conjugated secondary antibodies were from Cell Signaling. Stealth-PON2 and control siRNAs (Invitrogen) sequences and methods have been previously described(22). 3OC12 and LPS were from Sigma.

Statistical analysis - Curve fitting and statistical analysis were performed with GraphPad Prism software.

RESULTS

PON2 mediates intracellular 3OC12-acid accumulation - We and others had previously observed that 3OC12-acid accumulated inside cells after treatment with 3OC12(11). This suggested that the hydrophobic 3OC12 is hydrolyzed inside the cell to its corresponding ring-opened 3OC12-acid, which is much more polar and likely unable to readily cross cellular membranes. We therefore compared the rates of intracellular accumulation of 3OC12-acid in human embryonic kidney cells (HEK) and HEK PON2 cells stably transfected with a human PON2-GFP construct. The HEK cells express low basal levels of PON2, 2.2 ± 0.2 U/mg, while the HEK PON2 cells have 70.4 ± 7.0 U/mg of PON2. When treated with 25 μM 3OC12, the rate of intracellular 3OC12-acid accumulation was much faster in the HEK PON2 cells and began to plateau rapidly, within 4 minutes (Fig 1A). The plateau is likely at least partly due to the ability of 3OC12 to cause a rapid inactivation of PON2 activity(23). We also compared the rates of intracellular accumulation of 3OC12-acid in a human endothelial cell line EA.hy 926 stably transfected with human PON2 (EA.hy PON2) or a human PON2 inactive H114Q mutant (EA.hy H114Q). The PON2 mutant retains its antioxidant and antiapoptotic functions, but does not have 3OC12 hydrolytic activity(22). Thus, this PON2 mutant is used to control for effects due to increased protein expression and any effects of PON2 not associated with its hydrolytic activity. We have also previously established that PON2 is the only enzyme that hydrolyzes 3OC12 in EA.hy cells(15). The EA.hy H114Q cells have 6.8 ± 0.4 U/mg of PON2 activity, due to significant basal levels of PON2, while the EA.hy PON2 cells have 25.8 ± 2.5 U/mg of PON2 activity. The rate of accumulation of 3OC12-acid in the EA.hy PON2 cells was significantly faster compared to the EA.hy H114Q cells and also began to plateau rapidly (Fig 1B). 3OC12 was not detected in the cells at any time point (detection limit 0.5 nmol/mg of cell lysate). These results demonstrate that PON2 mediates the 3OC12-acid accumulation within the cells.

3OC12 causes a PON2-dependent cytosolic acidification and calcium release - The rapid intracellular accumulation of 3OC12-acid suggested a potential corresponding decrease in intracellular pH (pHi). Therefore, we directly visualized the change in pHi in naïve (non-transfected) EA.hy cells after 3OC12 treatment by laser scanning confocal microscopy. Because decreased pHi can induce Ca^{2+} release and increased cytosolic Ca^{2+} $[Ca^{2+}]_c$ is a common response to 3OC12 in mammalian cells(5;24), we also concomitantly measured $[Ca^{2+}]_c$ fluxes. The cells were simultaneously loaded with the Ca^{2+} indicator Fluo-4 AM and the pH indicator SNARF-4F, treated with 3OC12, and time lapse images acquired. As shown in figure 1C and D, 3OC12 caused a very rapid decrease in pHi and increase in $[Ca^{2+}]_c$. Interestingly, the rapid pHi decrease appeared to return to initial levels within 4 minutes, but then decreased again before slowly returning to initial levels (Fig 1D). The decrease in pHi appeared to just precede the increase in $[Ca^{2+}]_c$, consistent with a potential intracellular acidification mediating Ca^{2+} release into the cytosol.

To assess the role of PON2 in the 3OC12-mediated pHi and $[Ca^{2+}]_c$ changes, PON2-expressing cells were loaded with the cytosolic pH indicator BCECF-AM or Fluo-4 AM, treated with 3OC12 and fluorescence measured on the microplate reader. In the HEK cells 3OC12 caused no detectable change in pHi and a slow, minor rise in $[Ca^{2+}]_c$ (Fig 1E and G). Conversely, in the HEK PON2 cells 3OC12 caused a rapid, almost immediate, decrease in pHi with a

concomitant pronounced rise in $[Ca^{2+}]_c$ (Fig 1E and G). Both pHi and $[Ca^{2+}]_c$ returned to initial levels by 10-15 minutes post treatment. 3OC12 also caused rapid decrease in pHi with a concomitant increase in $[Ca^{2+}]_c$ in the EA.hy H114Q and EA.hy PON2 cells (Fig 1F and H). However, compared to the EA.hy H114Q cells, the pHi decrease and the Ca^{2+} increase in the EA.hy PON2 cells were significantly greater. Also, the increase in Ca^{2+} was delayed in the EA.hy H114Q cells compared to the EA.hy PON2 cells. Thus, within each cell type the extent of intracellular acidification and Ca^{2+} release corresponded closely with the time course of 3OC12-acid accumulation and cellular PON2 levels. The pHi and $[Ca^{2+}]_c$ changes in the EA.hy H114Q and EA.hy PON2 cells were also transient, lasting 20-25 minutes.

3OC12 causes a PON2-dependent phosphorylation of MAPK p38 and eIF2α - p38 and eIF2α are kinases that are activated in response to stressors. Activated eIF2α inhibits protein translation and is a marker of ER stress, and phosphorylation of both p38 and eIF2α are established immediate responses to 3OC12(8). Furthermore, p38 is phosphorylated in response to intracellular acidification(25). Therefore, we hypothesized the phosphorylation of these kinases by 3OC12 treatment would be dependent on PON2. Compared to naïve EA.hy cells and EA.hy H114Q cells the EA.hy PON2 cells exhibited increased p38 and eIF2α phosphorylation in response to 3OC12 (Fig. 2B-D). Phosphorylation of p38 by LPS was the same in the EA.hy naïve, H114Q and PON2 cells demonstrating that PON2 only affects 3OC12-mediated p38 phosphorylation (Fig. 2B-D). To see if such effects were also dependent on PON2 in primary cells, phosphorylation of p38 and eIF2α was investigated in primary human umbilical vein endothelial cells (HUVECs). 3OC12 treatment resulted in a significant dose-dependent phosphorylation of p38 and eIF2α (Fig. 2A). Decreasing PON2 levels by RNAi also diminished the 3OC12-mediated p38 and eIF2α phosphorylation in the HUVECs (Fig 2E and F). Collectively, the data demonstrate the 3OC12-mediated phosphorylation of p38 and eIF2α are dependent upon PON2.

DISCUSSION

3OC12 elicits a spectrum of biological effects in diverse host cell types, many of which are believed to favor *PA* persistence. While 3OC12 can activate the T2R38 receptor in sinonasal cells, and modulate PPAR receptor activities, the mechanism mediating many of 3OC12's effects remains enigmatic(10;12). Here we demonstrate that $[Ca^{2+}]_c$ increase and phosphorylation of p38 and eIF2-α, common immediate biological effects of 3OC12, are dependent upon intracellular PON2-mediated hydrolysis of 3OC12 to its 3OC12-acid. The rate and time course of 3OC12-acid accumulation corresponds closely with the time course of cytosolic acidification and the measured biological responses. Thus, our data support a novel mechanism in which PON2 hydrolyzes 3OC12 to its carboxylic acid, which becomes trapped within the cell, causing an intracellular acidification triggering pH-dependent biological responses.

We propose that intracellular acidification is the initial event mediating our observed biological responses to 3OC12. Phosphorylation of p38 by 3OC12, demonstrated to be dependent on PON2 in this study, is known to be induced by intracellular acidification(25). Many proteins that have critical roles in biological functions are very sensitive to minute changes in pH(26). Therefore, cells stringently regulate pHi by employing diverse families of proton extruders and pumps and a balance of intracellular weak acids and bases to increase buffering capacity(26). This propensity of cells to maintain pH homeostasis is exemplified in our cell lines, where pHi decreases return to initial levels within 10-20 minutes after 3OC12 treatment (Fig. 1E and F). The pH indicator used in this study, BCECF-AM, is retained in the cytosol and is a measure of pH in this compartment. However, PON2 is predominantly localized to the inner mitochondrial membrane and likely the lumen of the ER. Therefore, upon 3OC12 treatment it is probable that 3OC12-acid is predominantly concentrating within these organelles and the modest pH drop we measured in the cytosol is a reflection of much greater pH decreases in the ER and mitochondria. Thus, we hypothesize that acidification within the ER and mitochondria is the proximal event mediating the pH-dependent biological responses to 3OC12.

Because PON2 is expressed in a wide variety of cells and tissues, most mammalian cells would be expected to hydrolyze 3OC12 intracellularly and potentially undergo acidification upon exposure to 3OC12. However, due to the array of pH regulating mechanisms within cells the response of different cell types to 3OC12-acid accumulation would not be expected to be the same(25;26). We found that the rate of intracellular 3OC12-acid accumulation in the HEK PON2 cells was slightly greater than that in the EA.hy PON2 cells, yet the pHi drop was less extensive and had a shorter time course (Fig 1 A, B, E, F). Interestingly, despite the less extensive pHi drop in the HEK PON2 cells, the maximal increase in $[Ca^{2+}]_c$ was greater and occurred earlier compared to the EA.hy PON2 cells (Fig 1 E-H). Thus, compared to the EA.hy PON2 cells the HEK PON2 cells appear to be more resistant to cytosolic pHi changes, but more sensitive to pHi-mediated $[Ca^{2+}]_c$ induction. Our findings suggest that, in addition to cellular PON2 levels, the ability of a given cell type to regulate pHi and the sensitivity of its signaling pathways to pHi changes will likely be critical factors modulating the response to 3OC12.

In summary this study demonstrates a novel PON2-dependent mechanism by which 3OC12 can elicit biological effects in mammalian cells. The PON2-mediated intracellular acidification occurs very rapidly and therefore likely contributes too many of the previously reported, yet unexplained, rapid cellular responses to 3OC12. This finding should greatly accelerate the understanding of how *PA*, as well as other gram-negative bacteria, utilize AHLs to modulate host immune responses.

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FIGURE LEGENDS

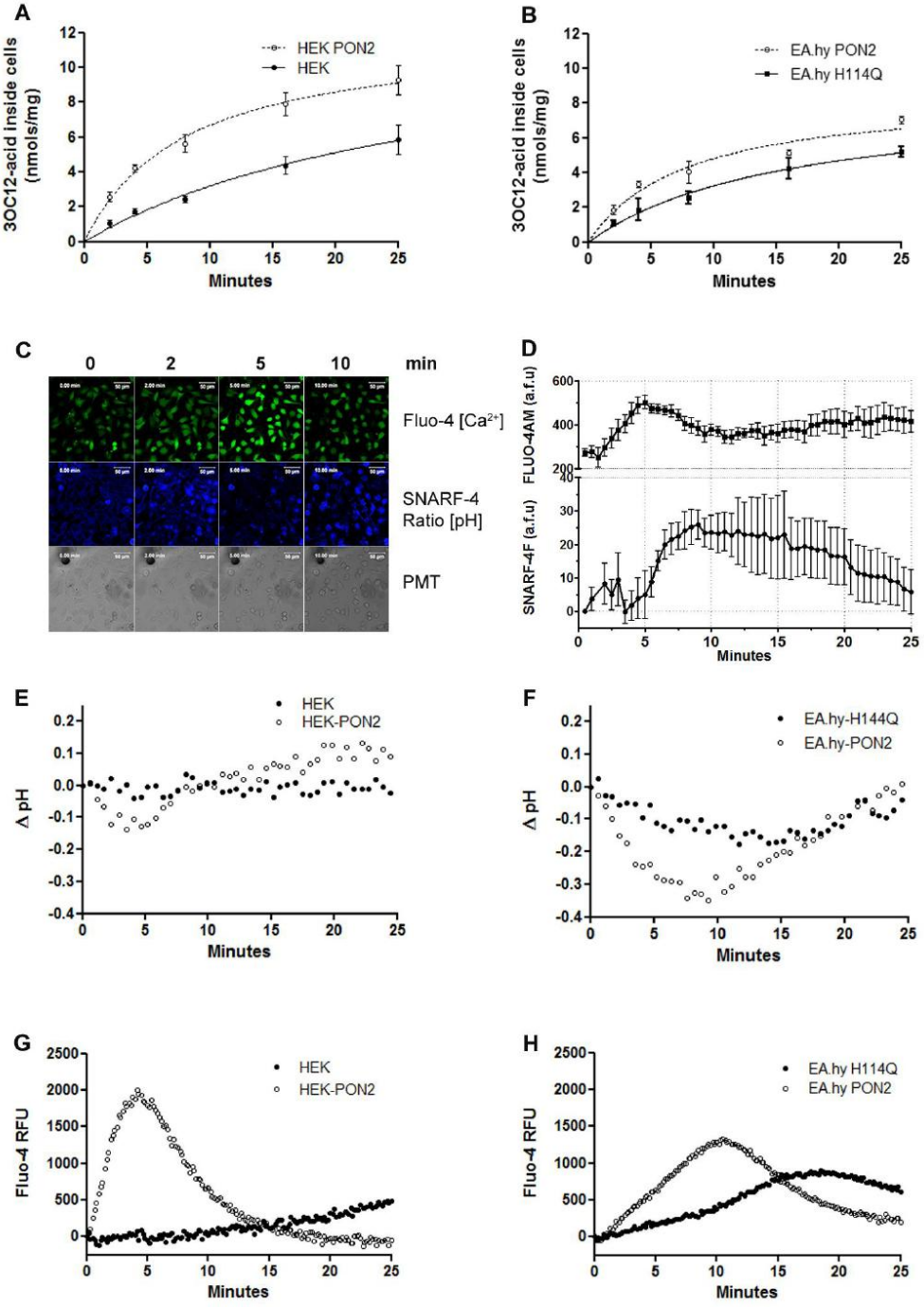
Figure 1

Intracellular 3OC12-acid accumulation, acidification and Ca^{2+} rise is PON2-dependent.
(A, B) HEK and EA.hy cells were treated with 25 µM 3OC12 and at shown times cells were rinsed, extracted with acetonitrile and extracts analyzed for 3OC12-acid by HPLC. The data points are the average of 3 separate treatments with the error bars representing the s.d. **(C, D)** Naive EA.hy cells were loaded with Fluo-4AM and SNARF-4F and fluorescence intensities recorded in confocal time-lapse images to monitor intracellular calcium and pH fluxes, respectively, in response to 50 µM 3OC12. Green channel (C; top; Fluo-4AM) reports cytosolic Ca^{2+} rise; intensity of blue channel (C; middle; ratio of acidic / basic SNARF-4F signals) is a measure of cytosolic acidification. Scale bar, 50µm (C). Fluorescence intensities for all cells were individually measured, allowing simultaneous determination of changes in pH and Ca^{2+} within the same cell. In D, SNARF-4F ratio before 3OC12 stimulation was set as zero. Graph shows mean / s.e.m. from 4 representative experiments. **(E-H)** HEK and EA.hy cells loaded with BCECF-AM to detect cytosolic pH values (E, F) or Fluo-4AM to detect cytosolic Ca^{2+} rise (G, H) were treated with 25 µM 3OC12 and fluorescence measured on the microplate reader. Shown is a typical trace from 4 independent experiments in which the background (DMSO treated) values were subtracted from the 3OC12 treated values (E-H).

Figure 2

3OC12-induced activation of p38 and eIF2a signalling is PON2-dependent.
(A) Primary HUVECs were treated with indicated concentrations of 3OC12 for 10min. Subsequently, levels of total and phosphorylated p38 and eIF2a were assessed by Western blotting. Tubulin served as loading control. **(B, C)** EA.hy cells were treated with 3OC12 (or LPS) as indicated, followed by immunoblotting against phosphorylated p38 and eIF2a. **(D)** Quantitative evaluation of p38 and eIF2a phosphorylation (fold) of 4 experiments as in B+C. **(E)** Similar experiment as in B+C, but using HUVECs that were untreated (naïve) or treated with PON2 specific or control siRNAs. **(F)** Quantitative evaluation of four experiments as in (E). Values for naïve and control siRNA treated cells was the same (not shown).

Figure 1


A
3OC12-acid inside cells (nmols/mg)
HEK PON2
HEK
Minutes
B
3OC12-acid inside cells (nmols/mg)
EA.hy PON2
EA.hy H114Q
Minutes
C
0
2
5
10
min
Fluo-4 [Ca2+]
SNARF-4 Ratio [pH]
PMT
D
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Minutes
F
Δ pH
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EA.hy-PON2
Minutes
G
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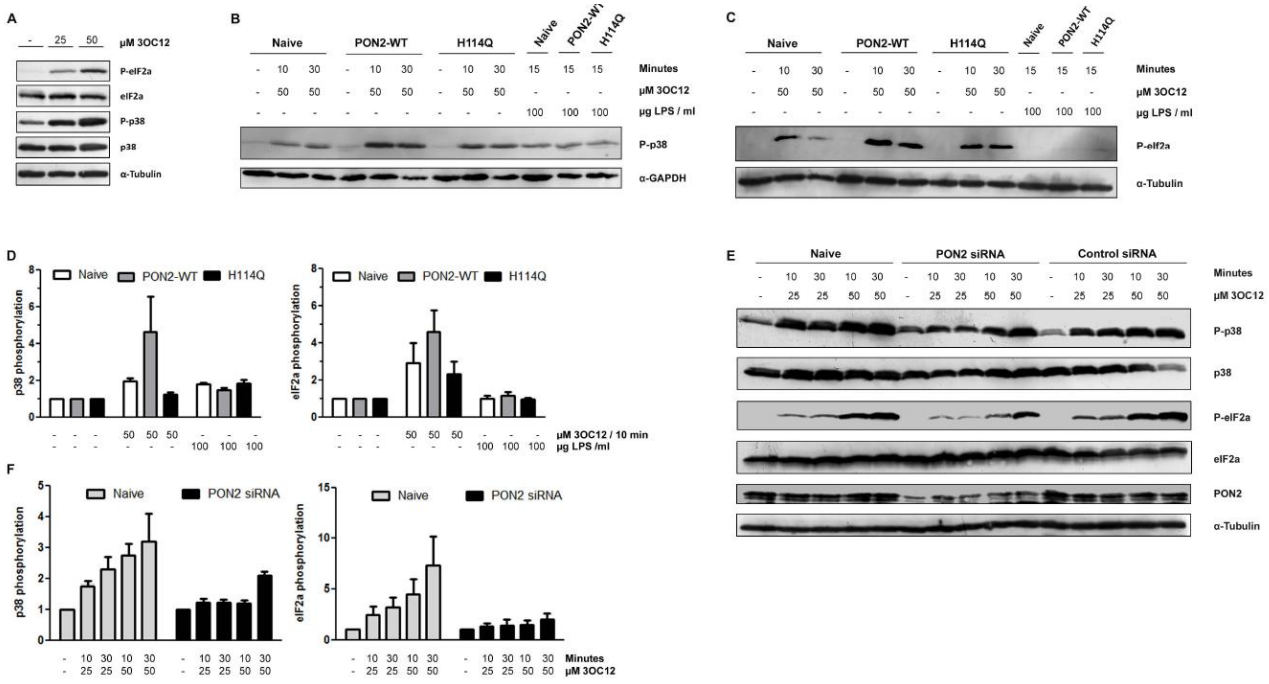
Minutes

Figure 2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B
C
D
E
F
Naive
PON2-WT
H114Q
PON2 siRNA
Control siRNA
μM 3OC12
Minutes
μg LPS / ml
P-eIF2a
eIF2a
P-p38
p38
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α-GAPDH
PON2
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eIF2a phosphorylation
μM 3OC12 / 10 min
μg LPS /ml

VIII. Supporting Data

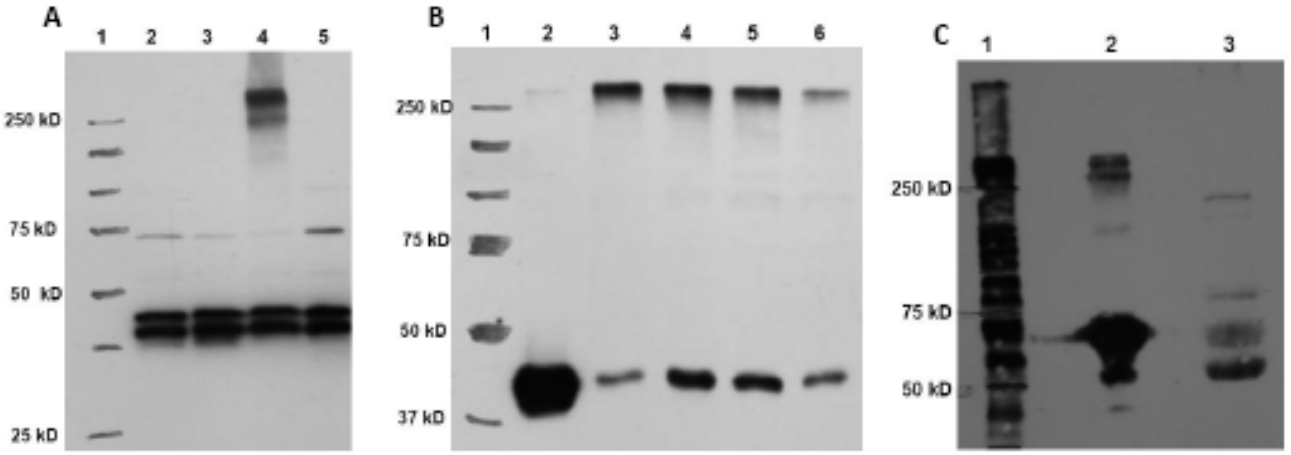

Figure 1. Western blots of crosslinked PON2. (A) A549 cells were untreated (lane 2), treated with 1.5% formaldehyde (lane 3), treated with 100 µM 3OC12 for 10 min followed by treatment with 1.5% formaldehyde (lane 4) or treated with 100 µM 3OC12 for 10 min (lane 5). Cells were then lysed and lysates analyzed by immunoblotting with an anti-PON2 antibody. Protein standards (lane 1). (B) A549 cells were untreated (lane 2), treated with HBSS pH 4.7 (lanes 3 and 4) or treated with 100 µM 3OC12 for 10 min. Cell were lysed and lysates treated with 0.5 mM of the crosslinking agent BS^3 and analyzed by immunoblotting with a PON2 antibody. For A and B PON2 is visible as band at ~45 kD and the PON2-crosslinked protein is visible as the ~300 kD band. Inactivation PON2 activity by 3OC12 and HBSS pH 4.75 was verified by HPLC analysis of lysates. (C) Lysates from EA.hy 926-PON2-GFP cells treated with 100 µM 3OC12 for 10 min were treated with 0.5 mM of the crosslinking agent BS^3. The crosslinked lysates were then immunoprecipitated with the anti-GFP antibody using anti-Rabbit IgG conjugated to magnets beads (Dynabead; Invitrogen) per the manufacturer's instructions and samples analyzed by immunoblotting with an anti-GFP antibody. Lane 2, immunoprecipitated PON2-crosslinked protein visible as a ~300kD band (arrow), lane 3, supernatant from the crosslinked sample. Protein standards lane 1

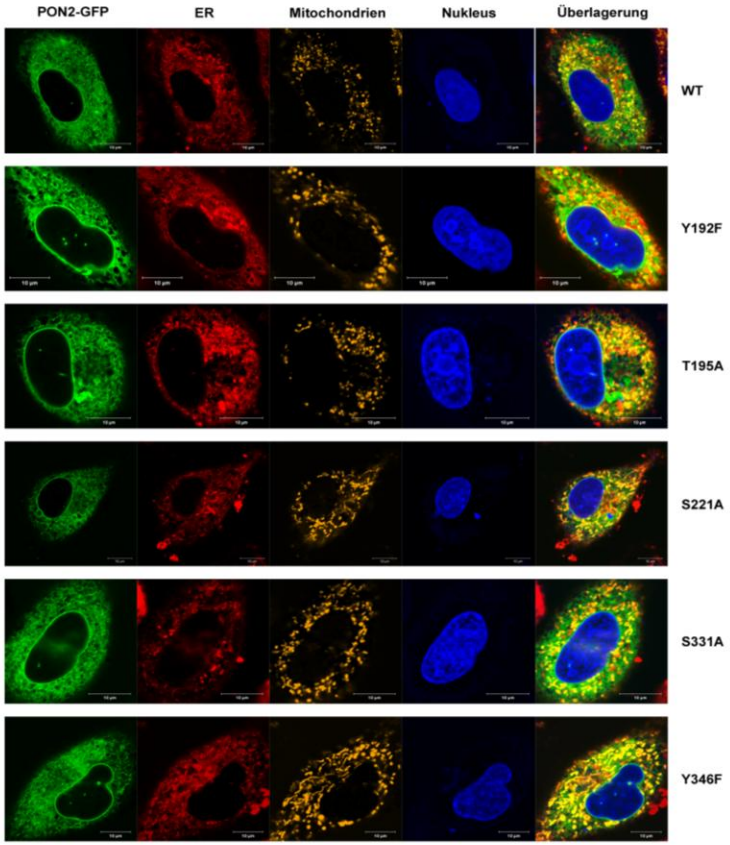

Figure 2. Confocal microscopy of PON2-GFP and PON2-GFP mutants. PON2-GFP and mutants are shown in green in column 1. ER is shown in red in column 2 and mitochondria and the nucleus are shown in orange and blue in columns 3 and 4, respectively. An overlay of all subcellular organelles and PON2 is shown in the last column.

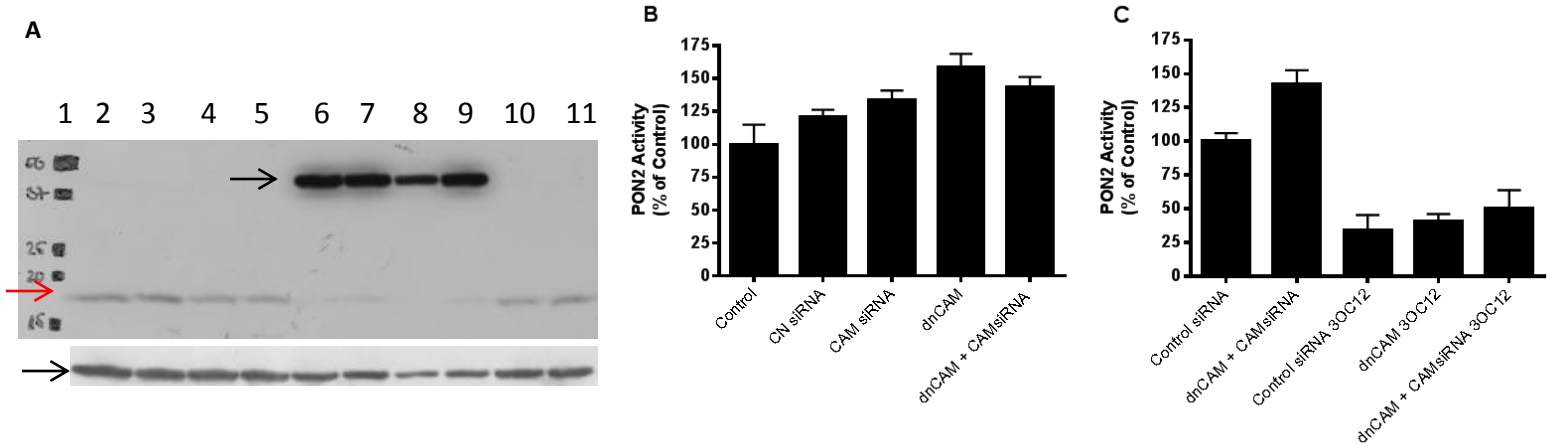

Figure 3. Effect of blocking calmodulin on cellular PON2 activity. EA.hy 926 cells were transfected with the dnCALM plasmid (using Nanofectin reagent) and/or three CALM siRNAs which target different regions of the RNA, or a control siRNA (using Synvolux, NL transfection reagent). Two days later, cells were either left untreated or treated with 75 μM 3OC12 for 10 min. (A) CALM expression in lysed cells was determined by immunobloting with a anti-human CALM antibody (Lifespan Bioscience). Lane 1- protein standards, lane 2- non-transfected; lane 3- 3OC12 only; lane 4- CALM siRNA ; lane 5- CALM siRNA+ 3OC12; lane 6; dnCALM; lane 7-dnCALM + 3OC12; lane 8- dnCALM + CALM siRNA; lane 9- dnCALM + CALM siRNA + 3OC12; lane-10 control siRNA; lane 11- control siRNA + 3OC12. Top black arrow, dnCALM; red arrow, endogenous CALM; bottom black arrow; tubulin loading control. (B) PON2 activity in cell lysates only transfected with calciunurin (CN) siRNA or combinations of dnCALM and CALM siRNAs. (C) PON2 activity in cell lysates transfected with combinations of dnCALM and CALM siRNAs followed by treatment with 3OC12. (A and B) the experiments were done in duplicate and error bars represent the range.

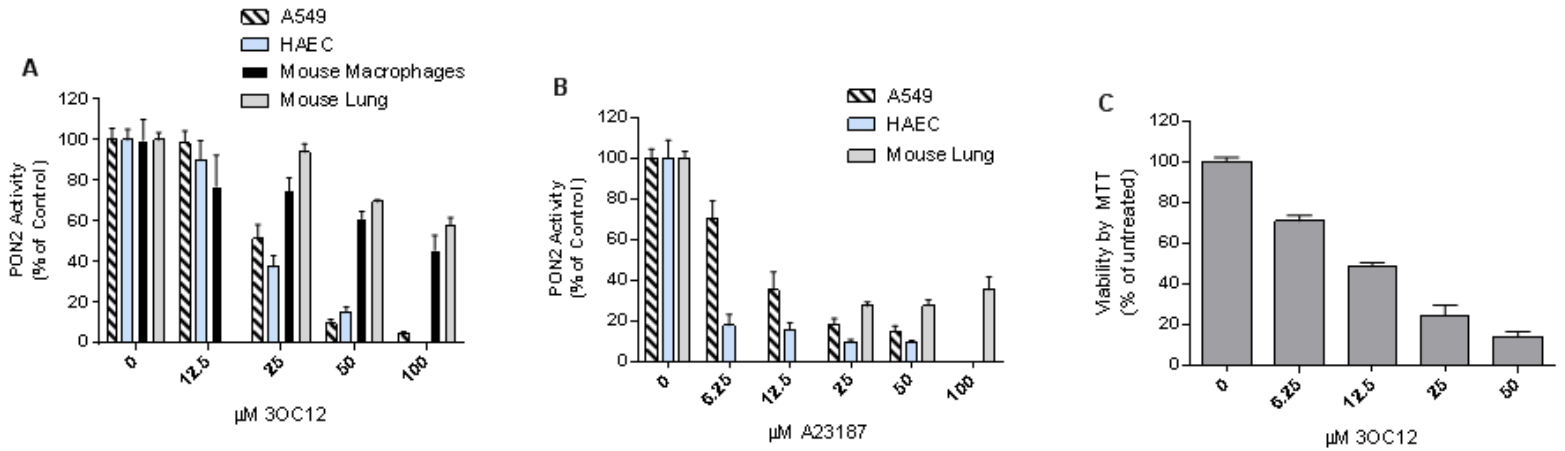

Figure 4. 3OC12-mediated PON2 inactivation and toxicity in primary human and mouse cells. Cells were plated in 24 well plates at 75,000 per well, except for macrophages which were plated in 48 well plates at about 75% confluency. Cells were treated for 10 min with 3OC12 (A) or the ionophore A23187 (B). Cell were then trypsinized pelleted and cell pellets lysed and analyzed for PON2 activity by HPLC. (C) HAEC cells were treated with 3OC12 for 16 hrs and cell viability determined by the MTT assay. All assays were done in triplicate and error the bars represent the SDs. A549-human epithelial lung cell line, HAEC-human aortic endothelial cells.

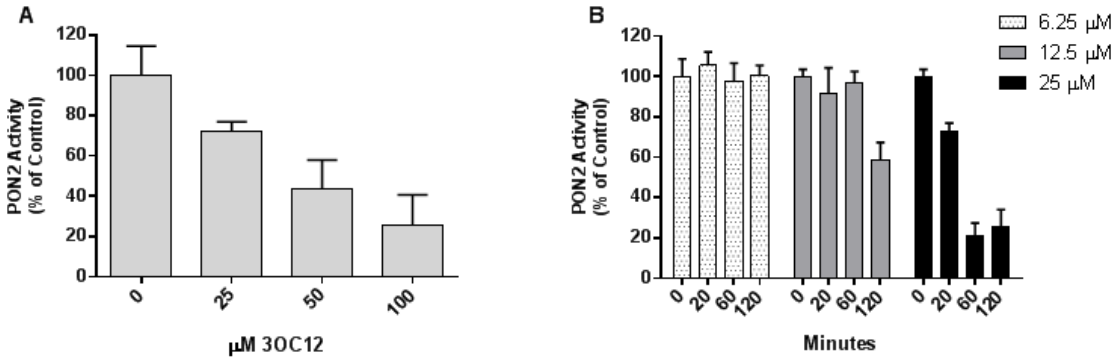

Figure 5. 3OC12-mediated PON2 inactivation in primary human bronchial epithelial cells. Cells were plated in 24 well plates at 75,000 per well and treated with 3OC12 for 10 min (A) or for increasing times (B). After treatment cells were trypsinized, pelleted and cell pellets lysed and analyzed for PON2 activity by HPLC. All assays were done in triplicate and error the bars represent the SDs.

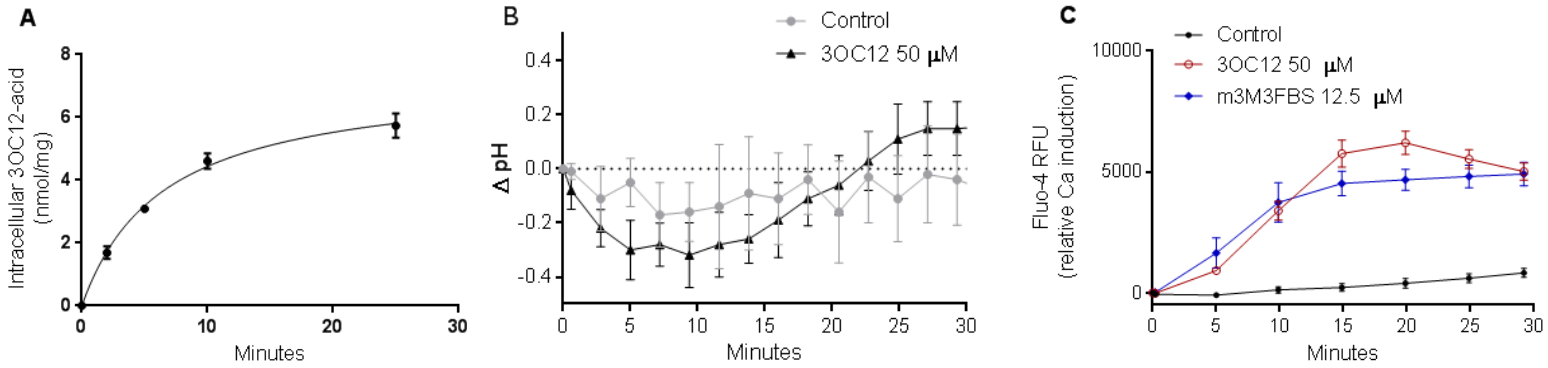

Figure 6. Biological effects of 3OC12 on primary human bronchial epithelial cells. (A) Cells were plated in 24 well plates at 75,000 per well and treated with 25 µM 3OC12 and cells then extracted with acetonitrile and extracts analyzed by HPLC for 3OC12-acid. (B) Cells were plated in 96 well plates at 25,000 per well, treated with 3OC12 and analzyed for cytosolic pH changes as described in the manuscript in the appendix. (C) Cells were plated in 96 well plates at 25,000 per well, treated with 3OC12 or the phospholipase C activator m3M3FBS and analyzed for cytosolic calcium release as described in the manuscript in the appendix. All assays were done in triplicate and error the bars represent the SDs.

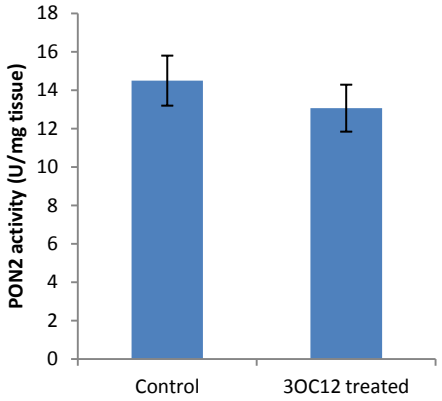

Figure 7. PON2 activity in mouse lung after 3OC12 treatment. Male mice were intubated with the Microsprayer and treated with 25 µl of 200 µM 3OC12 in water containing 0.25% evans blue or treated with 0.25% evens blue only (control). After 30 minutes, mice were perfused and lungs harvested. Lung sections stained blue were excised, homogenized and analyzed for PON2 activity. Each group contained 5 mice. Error bars, SDs.